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Introduction:

The Hepatocyte Nuclear Factor-3 (HNF-3) family of *forkhead* transcription factors (HNF-3α, β and γ) are essential for endoderm development (Kaestner et al., 1994). HNF-3 proteins, along with other cell specific transcription factors, have been shown to activate the transcription of numerous genes important for hepatocytic and respiratory epithelial cell function (Kaestner et al., 2000). We have recently demonstrated that HNF-3α is a transcriptional potentiator for androgen-regulated prostatic gene expression in epithelial cells and is also necessary for normal murine prostate development (Gao et al., 2003; Mirosevich et al., 2005; Gao et al., 2005). Further, I have obtained significant correlative evidence for the role of HNF-3α and β proteins in oncogenic involvement in tumor initiation and/or progression, in addition to prostate epithelial differentiation (Mirosevich et al., In Press). HNF-3α and β protein expression is altered in both the LADY transgenic mouse prostate cancer model, in human prostate adenocarcinomas and is differentially expressed in a variety of well established human prostate cancer cell lines (Mirosevich et al., In Press). The precise role that HNF-3 proteins play in normal prostate epithelial cell function, prostate cancer development and progression is unknown.

The signal transducer and activator of transcription-3 (Stat3) protein belongs to the Stat family of transcription factors and regulates numerous genes involved in normal cellular processes including cell proliferation, differentiation and apoptosis (Yu & Jove, 2004). In normal cells Stat3 phosphorylation is tightly regulated, however during cellular transformation Stat3 expression becomes altered and is frequently observed to be up-regulated and constitutively activated, i.e. phosphorylated, in many tumors, including prostate carcinomas (Mora et al., 2001). HNF-3α and Stat3 have previously been shown to interact directly and bind to the core enhancer region of the hepatitis B virus to regulate viral gene transcription (Waris & Siddiqui, 2002). I have recently identified and confirmed that Stat3 transcription factor binding sites are located adjacent to HNF-3 sites in the promoters of several prostate specific genes including the Prostate Specific Antigen (PSA) gene. Currently, the roles that HNF-3α and Stat3 protein interactions play in regulating normal prostate specific gene transcription, epithelial cell function and tumor initiation/progression are unknown.

The overall objective of this proposal is to elucidate the roles that HNF-3 and Stat3 proteins play in normal and cancerous prostate epithelial cells by employing several molecular biological techniques; *in vitro* and *in vivo* models. The first goal is to establish the functional significance of HNF-3α and β proteins, in normal and cancerous prostatic epithelial cells. The central hypothesis of this project is that HNF-3α and β are involved in prostatic epithelial differentiation and that altered expression of these genes is important in development and/or progression of prostate adenocarcinoma. The second goal is to establish the functional significance of HNF-3α and Stat3 interactions in normal and cancerous prostatic epithelial cell gene regulation. The hypothesis is that HNF-3α and Stat3 interact to regulate genes involved in normal prostatic epithelial differentiation and that the expression and interaction of these transcription factors is important in the development and/or progression of prostate adenocarcinoma. In summary, the proposed research will define the importance of HNF-3 *forkhead* proteins and Stat3 proteins in prostate epithelial cells and the development/progression of prostate tumors.
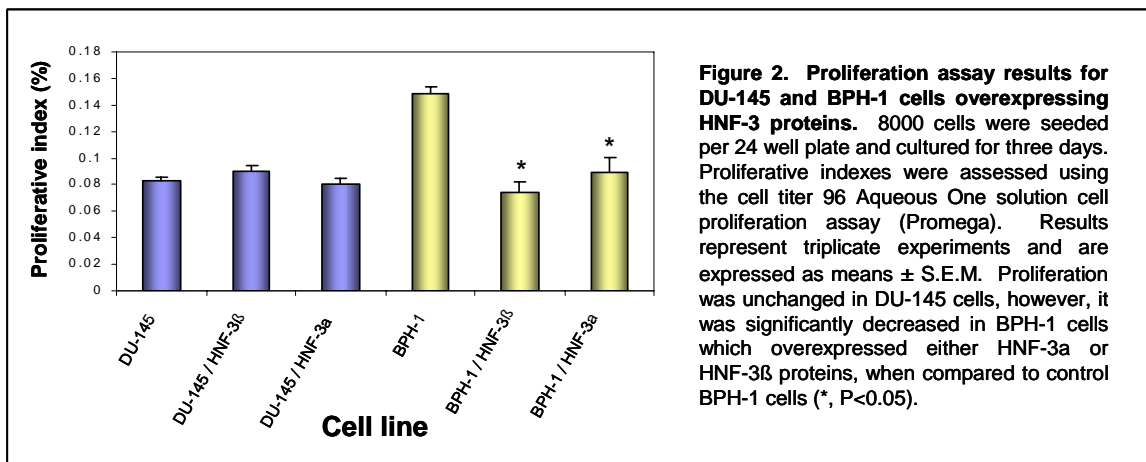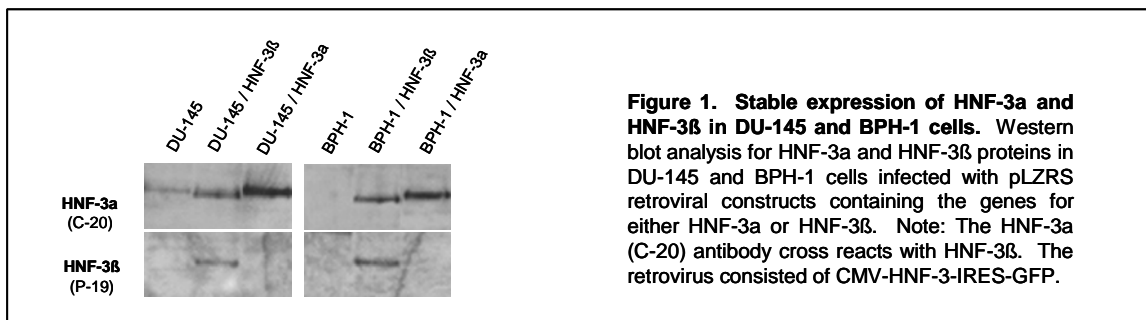
Task 1.

Research Performed

- While transient transfection experiments, using a tetracycline inducible system and tetracycline regulated HNF-3α siRNAs, produced adequate protein knockdown, similar experiments performed to test siRNAs towards HNF-3β failed to produce a decrease in protein levels. As such, experiments using Dharmacon RNAis directed towards HNF-3 were performed. Unfortunately, these siRNAs also failed to adequately reduce HNF-3β protein levels (results not shown).
- Due to the problems associated with the *in vitro* RNAi knockdown experiments, Task 1.5: Vary the ratios of HNF-3α and β in epithelial cells for characterization, was not pursued further.

- DU-145 and BPH-1 prostate cancer cells lines were retrovirally infected with pLZRS-CMV-HNF-3α or HNF-3β expression vectors to produce HNF-3 overexpressing prostate cancer cells (Figure 1). LNCaPs could not be infected with the HNF-3β expression construct. As such, a lentiviral expression construct is being created to infect these cells.
- The proliferation rates of control and infected DU-145 and BPH-1 cell lines were measured using the cell titer 96 Aqueous One solution cell proliferation assay. The apoptosis assay has yet to be performed.
- Western blot analysis was performed on the cell lines to examine *in vitro* androgen receptor, PSA and synaptophysin expression.

Key Research Findings

- Overexpression of HNF-3α and β (Figure1), had no effect on the *in vitro* proliferative index of DU-145 cells, but did increase proliferation of BPH-1/HNF-3 infected cells (Figure 2). BPH-1 cells overexpressing HNF-3 proteins were chosen for subsequent tissue recombinant experiments.
- No differences in androgen receptor, PSA and synaptophysin expression were observed between experimental and control DU-145 or BPH-1 cell lines (results not shown).


Figure 1. Stable expression of HNF-3a and HNF-3ß in DU-145 and BPH-1 cells. Western blot analysis for HNF-3a and HNF-3ß proteins in DU-145 and BPH-1 cells infected with pLZRS retroviral constructs containing the genes for either HNF-3a or HNF-3ß. Note: The HNF-3a (C-20) antibody cross reacts with HNF-3ß. The retrovirus consisted of CMV-HNF-3-IRES-GFP.

Figure 2. Proliferation assay results for DU-145 and BPH-1 cells overexpressing HNF-3 proteins. 8000 cells were seeded per 24 well plate and cultured for three days. Proliferative indexes were assessed using the cell titer 96 Aqueous One solution cell proliferation assay (Promega). Results represent triplicate experiments and are expressed as means ± S.E.M. Proliferation was unchanged in DU-145 cells, however, it was significantly decreased in BPH-1 cells which overexpressed either HNF-3a or HNF-3ß proteins, when compared to control BPH-1 cells (*, $P<0.05$).

Task 2.

Research Performed

- HNF-3α or HNF-3β overexpressing BPH-1 cell lines were recombined with rat embryo 18 day urogenital sinus mesenchymal cells and placed under the kidney capsule of athymic nude mice.
- Eight mice were used for 16 experimental grafts, containing cells over expressing HNF-3α or HNF-3β, in the left kidney. Twelve BPH-1 tissue recombination controls were grafted into the right kidney.
- Mice were sacrificed at six weeks and three months after implantation, the grafts harvested and analyzed for histology, HNF-3 expression, proliferation and expression of various differentiation markers, i.e. androgen receptor, PSA and synaptophysin. Immunohistochemical staining for apoptosis has yet to be performed.
- The proposed tumorgenicity studies have not been performed due to my recent move from the Moffitt Cancer Center in Tampa, Florida to the Beckman Research Institute of the City of Hope in Duarte, California. (see Additional Comments, page 10)

Key Research Findings

- Histologically, the HNF-3α and HNF-3β infected BPH-1 tissue recombinant grafts appeared similar to control BPH-1 cells. All tissue recombinants appeared to form canalized glandular epithelial structures with defined eosinophilic basement membrane surrounded by stroma. (Figure 3).
- As expected, BPH-1 infected cells expressed their respective HNF-3 proteins (Figure 4A&B). No differences were observed in the number of proliferating epithelial cells between experimental and control groups (results not shown). Interestingly, the control non-HNF-3 infected BPH-1 tissue recombination grafts also expressed HNF-3α (Figure 4C), but not HNF-3β (results not shown).
- When BPH-1 cells were grafted under the renal capsules without mesenchymal cells, the SV-40 transformed BPH-1 cells expressed T-antigen, however, HNF-3α expression was not detected by immunohistochemistry (Figure 5B).
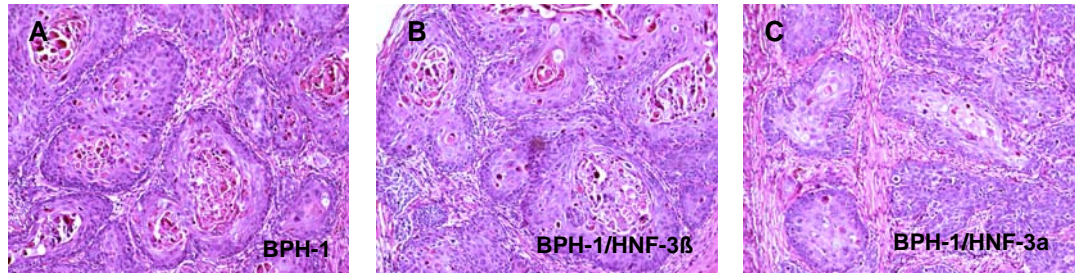
Figure 3. Histology of BPH-1 and BPH-1 HNF-3 tissue recombinants. The epithelial glandular structures which formed in the **(B)** BPH-1/HNF-3ß and **(C)** BPH-1/HNF-3a tissue recombinants were similar to the glandular structures which formed in the **(A)** BPH-1 control tissue recombinants. x10
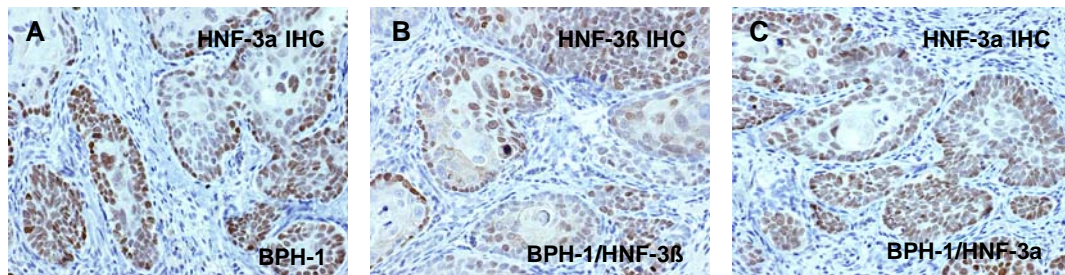

Figure 4. Immunohistochemical (IHC) analysis for HNF-3 expression in BPH-1 tissue recombinants following 6-weeks of sub-renal grafting. (B) Strong HNF-3ß expression was detected in BPH-1 epithelial cells infected with HNF-3a, while **(C)** strong HNF-3a expression was detected in BPH-1/ HNF-3a infected cells. **(A)** Interestingly, HNF-3a expression was also detected in epithelial cells of non-infected control BPH-1 tissue recombinants. x20 All cells expressed T-antigen while BPH-1/ HNF-3a and BPH-1/ HNF-3ß also expressed GFP (results not shown).
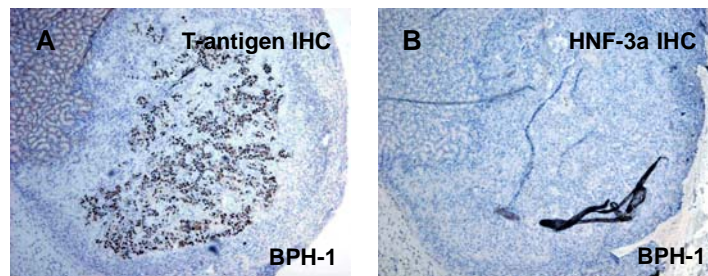
Figure 5. Immunohistochemical (IHC) analysis for T-antigen and HNF-3 expression in BPH-1 cells following 4-weeks of sub-renal kidney capsule grafting. Immunohistochemical staining of serial sections derived from kidney capsule grafts of BPH-1 cells without urogential sinus mesenchyme tissue recombination showed; **(A)** Strong T-antigen expression was observed in BPH-1 epithelial cells, however, HNF-3a expression was not detected in these grafts **(B)**. x5

- We have previously demonstrated that HNF-3β is expressed in synaptophysin expressing neuroendocrine cells of normal mouse (Figure 6A) and human prostates; neuroendocrine prostate tumors from the highly metastatic androgen independent 12T-10 LADY prostate cancer model (Figure 6B) and small cell carcinomas from human prostate biopsy samples (Mirosevich et al., In Press).
- Synaptophysin expression was examined in BPH-1/HNF-3β tissue recombination experiments by immunohistochemistry, however, no synptophysin expression was detected in the tissue recombinant samples (Figure 7).
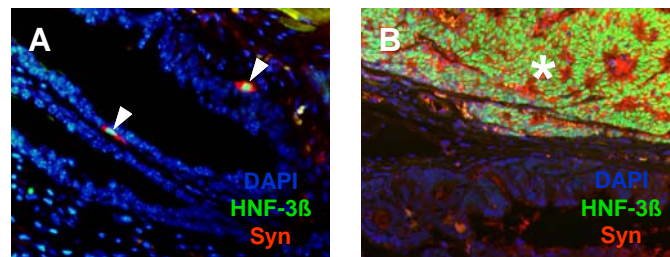
Figure 6. Dual HNF-3β and synaptophysin (Syn) immunofluorescence. (A) In the normal adult prostate, HNF-3β expression was exclusively localized to individual basal epithelial cells of the periurethral ducts which also co-expressed the neuroendocrine cell marker synaptophysin (Syn, arrowheads) x40 (Mirosevich et al., 2005). **(B)** Dual HNF-3β-synaptophysin expression was also observed in neuroendocrine prostate tumors (*) which formed in the 12T-10 transgenic LADY mice prostate cancer model x20 (Mirosevich et al., *In Press*). DAPI served as a counterstain.
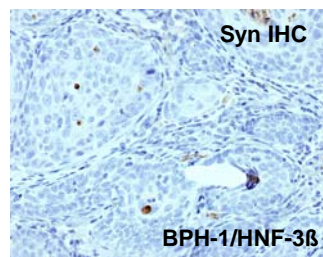
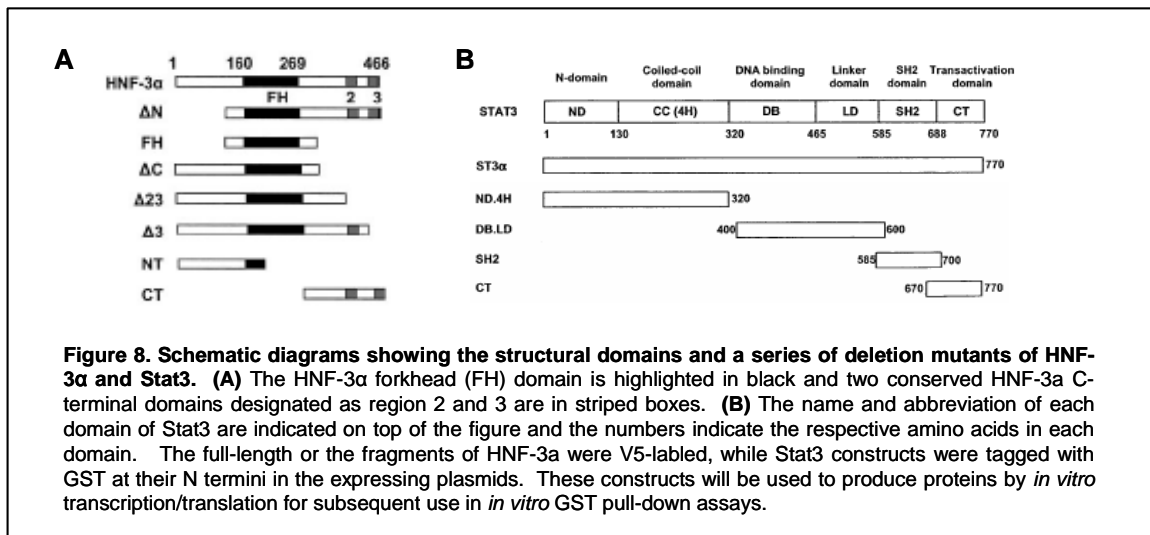**Figure 7. Synaptophysin expression in BPH-1/HNF-3β tissue recombinants following 6-weeks of sub-renal grafting.** Immunohistochemical analysis did not detect any synaptophysin expression in the BPH-1/HNF-3β expressing epithelial cells. x20. Androgen receptor and prostate specific antigen (PSA) expression was also absent in these samples and the BPH-1/HNF-3a tissue recombinants (results not shown).

Task 3.

Research Performed

- I have recently shown, by electromobility shift assay (EMSA), that Stat3 binding sites exist adjacent to HNF-3α on prostate specific promoters. As such, I plan to perform experiments to determine how these two proteins interact to regulate prostate gene transcription.
- I have acquired both full-length and various truncated HNF-3α-V5 tagged expression constructs that have previously been used to determine HNF-3 and androgen receptor interactions (Figure 8A; Gao et al., 2003).
- DU-145 prostate cancer cell lines, which constitutively expresses activated Stat3, were transfected with these HNF-3α-V5 tagged expression constructs. A preliminary co-immunoprecipitation experiment sshowed that Stat3 interacts with the *forkhead* DNA binding domain of HNF-3α.
- Recently, full length and truncated Stat3-GST tagged constructs have been kindly donated by Dr. Xinmin Cao (Figure 8B; Zhang et al., 2002). These constructs will be transcribed and translated *in vitro* and used in GST-pull down experiments. Protein interactions will be determined by western blot analysis using the V5 antibody (Invitrogen, Carlsbad, USA).

Figure 8. Schematic diagrams showing the structural domains and a series of deletion mutants of HNF-3α and Stat3. **(A)** The HNF-3α forkhead (FH) domain is highlighted in black and two conserved HNF-3a C-terminal domains designated as region 2 and 3 are in striped boxes. **(B)** The name and abbreviation of each domain of Stat3 are indicated on top of the figure and the numbers indicate the respective amino acids in each domain. The full-length or the fragments of HNF-3a were V5-labled, while Stat3 constructs were tagged with GST at their N termini in the expressing plasmids. These constructs will be used to produce proteins by *in vitro* transcription/translation for subsequent use in *in vitro* GST pull-down assays.

Additional Comments

- In September of this year I moved with Dr. Jove from the Moffitt Cancer Center in Tampa, Florida to the Beckman Research Institute of the City of Hope in Duarte, California. Subsequently, I received a promotion from a post-doctoral fellow to research assistant scientist.

Conclusions

The findings from these studies have provided interesting insights into the biological and molecular roles of HNF-3 and Stat3 proteins in prostate cancer cells. Results from the last six months have demonstrated several key findings. Firstly, *in vitro* cell culture conditions are quite different to the *in vivo* conditions that can be achieved by techniques such as tissue recombination. These differences were highlighted when the BPH-1 cell *in vitro* proliferation results were compared with the *in vivo* tissue recombination experiments; in addition to the re-expression of HNF-3α following tissue recombination and sub-renal grafting. The re-expression of HNF-3α following tissue recombination with urogenital sinus mesechymal cells also confirms that epithelial-stromal interactions play an important and necessary role in prostate organogenesis (Marker et al., 2003). Classical tissue recombination experiments by Cunha and colleagues have established that epithelial and stromal cells produce reciprocal paracrine signaling molecules to induce normal prostate development (Cunha et al., 1987). The findings from my work show, for the first time, that mesenchymal/stromal cells produce signaling molecules that stimulate expression of HNF-3α, an important transcription factor for both normal prostate development and cancer. The signaling molecules, which are involved in stimulating the *in vivo* HNF-3α epithelial cell expression, are currently unknown and are actively being investigated.

In my initial research proposal, I expected that the expression of HNF-3β may cause luminal epithelial cells to differentiate into a neuroendocrine phenotype. However, the tissue recombination results in this study demonstrated that HNF-3β alone is insufficient to promote a prostatic neuroendocrine epithelial cell phenotype, suggesting and that other factors are required. Since, BPH-1 cells do not express the androgen receptor (Wang et al., 2001), all the BPH-1 cell sub-lines produced thus far have been infected with androgen receptor expressing constructs. These cells will be used in subsequent tissue recombination experiments to see the effects of HNF-3 and androgen receptors on prostate epithelial cell biology/phenotype.

Finally, the preliminary immunoprecipitation experiments performed show that the *forkhead* DNA binding domain directly interacts with Stat3. This interaction is similar to our previously published work demonstrating that the androgen receptor also interacts with the DNA binding domain of HNF-3α (Gao et al., 2003), and suggests that the androgen receptor/HNF-3α/Stat3 proteins associate within close proximity to form a prostate tissue specific transcriptional unit.

The initiation and progression of prostate cancer is considered a multi-step process involving, primarily, dysregulated gene expression. Currently, very little is known about the genes which are necessary for this process. Elucidating the role of HNF-3 and Stat3 protein interactions in both prostate gene regulation and tumor initiation and/or progression will define the importance of HNF-3/Stat3 transcription factors in prostate biology, and will verify that these protein complexes are potential therapeutic targets for the treatment of advanced prostate cancer.

Training

So far this year I have attended two national meetings.
- American Association for Cancer Research 96th Annual Meeting, Anaheim, CA, April 2005.
- Society for Basic Urologic Research Spring 2005 Meeting, San Antonio, TX, May 2005.

I will also attend and participate in a third national meeting in December.
- Society for Basic Urologic Research Fall 2005 Meeting, Miami, FL, December 2005.

In addition, I gave an oral presentation to the Department of Pathology at Royal Perth Hospital in Perth, Western Australia, during a visit to Australia in February.

Lunchtime and evening seminars and mini symposiums also contributed to an overall training strategy in place within the Moffitt Cancer Center and the Department of Molecular Oncology. Finally, laboratory meeting discussions and journal club presentations are integral to the running of the department. I attended and presented within these forums.

Reportable outcomes

Publications

Mirosevich J, Gao N, Gupta A, Shappell SB, Jove R & Matusik RJ. 2005 The expression and role of Foxa proteins in prostate cancer. *The Prostate,* In Press.

Nam S, Kim D, Cheng JQ, Zhang S, Lee J, Buettner R, **Mirosevich J**, Lee FY & Jove R. 2005 Action of the Src family kinase inhibitor, Dasatinib (BMS-354825), on human prostate cancer cells. *Cancer Research,* **65** 9185-9189.

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Poster Abstracts

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